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REPETITIVE MOLECULAR EXCLUSION CHROMATOGRAPHY OF PGB_X ON SEPHADEX LH-20

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PGB_x, a mixture of oligomers of 15-keto PGB_1, was subjected to repetitive MEC on Sephadex LH-20 in an attempt to separate and purify the active principle. The resulting fractions were analyzed and found still to be hetereogeneous. Although the MEC technique did not resolve the PGB_x complex, low molecular weight fractions were obtained that may be amenable to mass spectral analysis.

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LIST OF ABBREVIATIONS

MEC - Molecular Exclusion Chromatography

$\bar{M}n$ - Number Average Molecular Weight

RLM - Rat Liver Mitochondria

Type II PGB_x - Fraction 2 of 1st Sephadex LH-20 MEC

VPO - Vapor pressure osmometry

INTRODUCTION

PGB_X, a polymeric derivative of 15-keto PGB_1, was first synthesized by Polis et al (1) and shown to conserve oxidative phosphorylation during degradation of isolated rat liver mitochondria (2). More recently Ohnishi and Devlin (3) showed that PGB_X behaved as a water soluble Ca^{++}-ionophore in skeletal muscle sarcoplasmic recticulum. Although these properties of PGB_X are significant, the major interest in this compound is its possible role as a therapeutic agent. This is based on the animal studies of (a.) Polis and Angelakos (4) in which PGB_X treated monkeys survived cardiogenic shock, (b.) Polis and Kolata (5) in which PGB_X treated rabbits survived experimentally induced global ischemia, and (c.) Moss et al (6) in which PGB_X treated dogs survived experimentally induced "fatal" hypoxia.

PGB_X prepared according to Polis et al is a mixture of oligomers of varying molecular weight and unknown chemical composition. Before human testing may be undertaken, the active principle in the PGB_X complex must be isolated in pure form and it chemical structure determined. Studies are currently underway in this and other laboratories to attain the above goal. This report describes the use of repetitive MEC of PGB_X on Sephadex LH-20 in order to obtain the PGB_X complex as a narrower molecular weight range of oligomers and thus a purer preparation.

MATERIALS AND METHODS

The PGB_X used in this study was prepared according to Polis et al (2) and was the crude extract before purification by MEC. When Sephadex LH-20 MEC was carried out, the procedure used was as described before (2). Molecular weights ($\bar{M}n$) were determined by VPO on the free acids dissolved in methanol, using a Wescan Molecular Weight Apparatus (Wescan, Santa Clara, CA) at 60°. Purified fractions were assayed for the in vitro PGB_X effect on the stabilization of oxidative phosphorylation during degradation of RLM by the method of Polis et al (7) as modified by Shmukler et al (8). This modification includes the definition and measurement of the PGB_X $\overline{K_a}$ and K_i effects. UV absorption spectra of PGB_X fractions were measured in a Cary Recording Spectrophotometer at a concentration of 30µg/ml dissolved in methanol.

RESULTS

In the original method for the preparation of PGB_X, Polis et al (2) used Sephadex LH-20 MEC in an attempt to purify the active principles of the PGB_X complex. Although the exclusion limit for Sephadex LH-20 is between 100-500 daltons, no clean chromatographic separations were obtained. Instead the PGB_X complex eluted as one diffuse chromatographic peak. In order to effect some degree of separation, Polis et al arbitrarily collected effluent fractions which were then monitored for their in vitro RLM oxidative phosphorylation effect.

The most active PGB_x preparation, fraction 2[1], gave a $\bar{M}n$ of 2200-2300 by VPO. Since this fraction was the PGB_x fraction sent to Office of Naval Research contractors for testing, it was of interest to try to purify this fraction even further. As a possible method to accomplish this, repetitive Sephadex LH-20 MEC was tried in order to purify the active principle and/or to obtain a PGB_x preparation with a narrower molecular weight range of components than the Type II PGB_x.

In this study 6620 mg of crude extract of PGB_x, i.e., the $NAHCO_3$ extract (2) were converted to the free acid, dissolved in methanol and chromatographed on Sephadex LH-20 as described by Polis et al (2). The resulting seven fractions were flash evaporated and analyzed for weight recovery, $\bar{M}n$ and in vitro PGB_x effects. These results are listed in table I. As seen in this table, fractions 1-2[2] and fraction 1-3 had approximately the same in vitro PGB_x activity, therefore these 2 fractions were combined in order to have sufficient material for further MEC and subsequent analyses. This combination contained 1932 mg of PGB_x of which 1385 mg were used in the 2nd MEC. Seven fractions were separated and analyzed as above. These results are listed in table II. As seen in this table fractions 2, 3, 4, and 5 all had approximately the same level of PGB_x activity, although the Mn values were markedly different. For the 3rd MEC fractions, 2-2 and 2-3 were combined (679 mg) and the analyses of the resulting seven fractions are listed in table III. The separation methodology used in this study is summarized by schematic representation shown in figure 1.

The overall weight recovery of PGB_x in the 1st MEC was 88.5 percent. This suggests that 11.5 percent of the crude extract was either irreversibly absorbed to the column, or adsorbed strongly to the column to elute outside the PGB_x range. The overall weight recovery of the 2nd and 3rd MEC was approximately quantitative, i.e., 95.7 and 101.9 percent respectively. This suggests that material not recovered in the 1st MEC is not PGB_x.

Chromatography of the crude PGB_x extract, 1st MEC, yields fractions varying in $\bar{M}n$ from 3049 to 372 as the retention time of the eluted material increased. Rechromatography of fractions 1-2 and 1-3 (2nd MEC) yielded fractions with $\bar{M}n$ varying from 2873 to 407. Even after 3rd MEC of fractions 2-2 and 2-3, the $\bar{M}n$ of the fractions varied from 2062-1466. Although the $\bar{M}n$ for fraction 3-7 could not be measured because of insufficient quantity, it would be reasonable to assume that the $\bar{M}n$ of fraction 3-7 would be similar to that of fraction 2-7.

The dry weights of all fractions separated in the repetitive MEC listed in tables I, II, and III were used to calculate the percent distribution of PGB_x in each fraction in each separate MEC. These results are listed in table IV. The percent distribution of PGB_x in the 1st MEC is approximately evenly distributed between fractions 1 through 6. On rechromatography of fractions 2-2 and 2-3, 59 percent of the PGB_x was found in fractions 3-2 and 3-3.

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[1]Fraction 2 of the 1st Sephadex LH-20 MEC is referred to as Type II PGB_x.

[2]Fractions separated by repetitive MEC are described by 2 numbers: 1st number refers to MEC No.; 2nd number refers to MEC fraction. Thus 1-2 describes fraction 2 from 1st MEC which is equivalent to Type II PGB_x.

The *in vitro* PGB_X assay data listed in tables I, II and III show that the K_a was *highest in* fractions 2, 3 and 4 of the 1st MEC. Fraction 1-1 was about 80 percent pure while fractions 1-5, 1-6, and 1-7 were relatively impure. Rechromatography of fractions 1-2 and 1-3 (2nd MEC) showed that the K_a was equally spread throughout fractions 2-2 to 2-6 with fractions 2-1 and 2-7 exhibition low values. When fractions 2-2 and 203 were rechromatographed (3rd MEC), only fractions 3-7 showed a low level of K_a. The results of the K_i distribution in the various fractions were similar to those found for the K_a distribution.

The UV absorption spectra between 200 nm to 400 nm were measured for all fractions separated in this study. In general, all fractions showed absorption maxima at 243 nm and absorption shoulders at 300- 320 nm. The major difference between the UV abosrption spectra of the separated fractions was the increase in the 300- 320 nm absorption shoulder with increasing retention time of the fractions. The results are listed in table V in terms of the ratio of the absorbance at 243 nm to absorbance at 310 nm. A comparison of the retention time and the A_{243}/A_{310} shows a progressive decrease (or increase in A_{310}) with increasing retention time. A comparison of the A_{243}/A_{310} of fractions with the same retention time but successive MEC, i.e., fractions 1-2 and fraction 2-2, shows a marked increase in the ratio (or a marked decrease in A_{310}). However, the data for the 2nd and 3rd MEC showed no change in the A_{243}/A_{310} for fractions with the same retention time.

D I S C U S S I O N

The results of repetitive MEC of PGB_X on Sephadex LH-20 shows that this method does not yield homogeneous PGB_X preparations as might have been expected. Instead, the fractions that were separated still appear to be hetereogeneous even after 3 MEC analyses. These results suggest that possibly even after additional MEC of fractions 3-2 and 3-3, homogeneous preparations of PGB_X would not be obtained. From these results it is obvious that Type II PGB_X, the preparation currently supplied ONR contractors for *in vitro* and *in vivo* animal studies, is a highly complex mixture of oligomers *varying in* $\bar{M}n$ *from* over 2800 to below 400 daltons. It is interesting to note also that the PGB_X fractions separated did not show a significant increase in the specific activity of the PGB_X, i.e., K_a.

One benefit realized with the repetitive MEC is that the PGB_X fractions separated in MEC #3 must have a narrower range of molecular weight components than found in Type II PGB_X. An additional advantage is the recovery of high K_a activity in relatively low molecular weight fraction, e.g. fraction 3-6 that had the following analytical values; $\bar{M}n$, 1466; K_a 0.93, K_i 0.92. It is conceivable that such a low molecular weight preparation may be amenable to high resolution and/or field desorption mass spectral analysis.

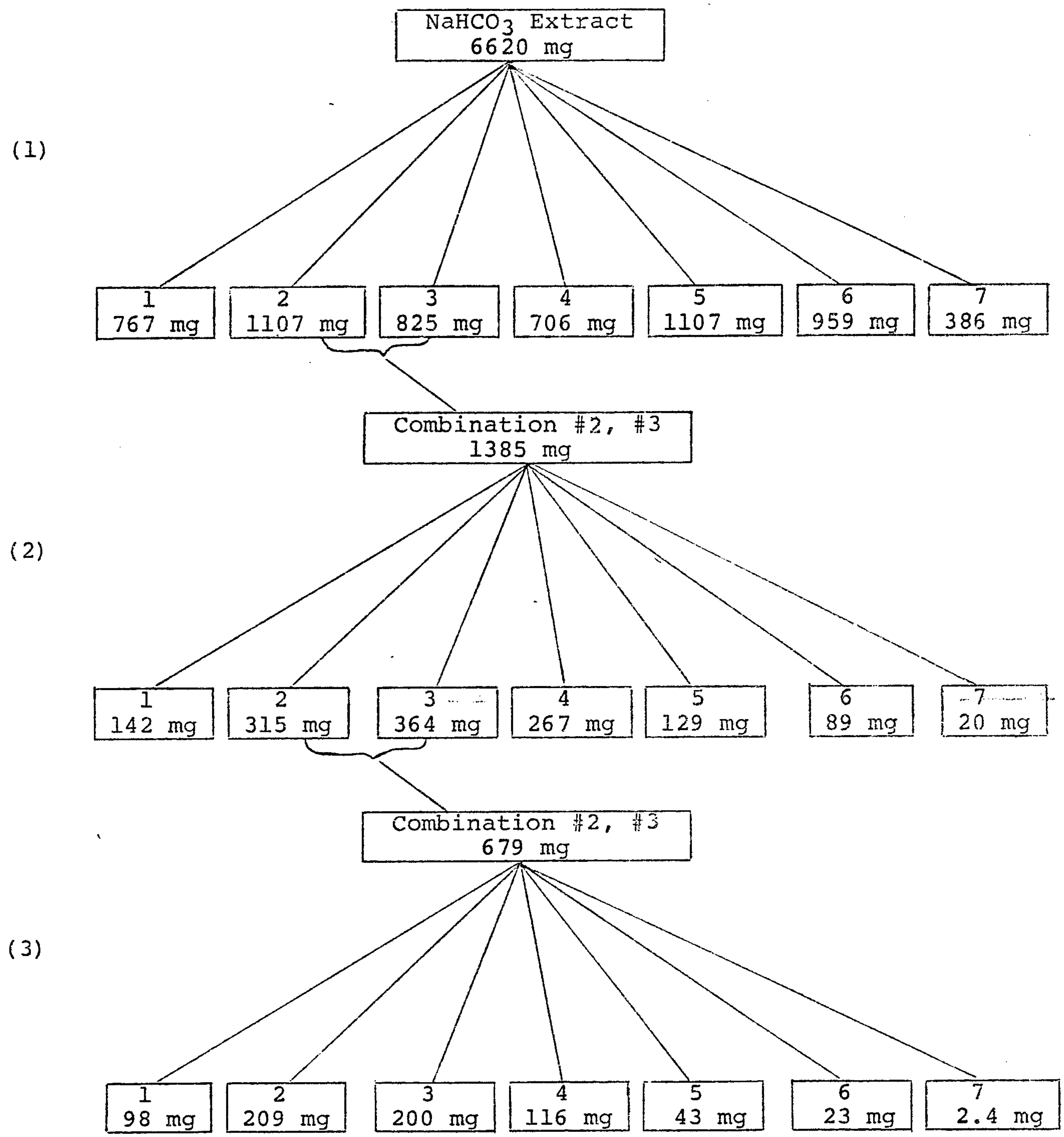

Figure 1 - Schematic Representation of Methodology for Repetitive MEC of PGB_x on Sephadex LH-20

Table I

Distribution of PGB_x in Sephadex LH-20

Fractions: 1st MEC

Fraction	$\bar{M}n$	Wt (mg)	K_a	K_i
1	3049	767	.82	1.18
2	2554	1107	.97	1.33
3	2137	825	1.02	1.48
4	1706	706	.98	1.36
5	1257	1107	.47	.81
6	915	959	.33	.40
7	372	386	.30	.12
Total Recovery	5858 mg			
%	88.5			

Table II

Distribution of PGB_x in Sephadex LH-20

Fractions: 2nd MEC

Fraction	$\bar{M}n$	Wt (mg)	K_a	K_i
1	2873	142	.31	.67
2	2683	315	.92	1.04
3	2351	364	1.06	1.08
4	2190	267	1.11	1.08
5	1919	129	1.08	1.06
6	1541	89	.93	.83
7	407	20	.53	.06
Total Recovery	1326 mg			
%	95.7			

Table III

Distribution of PGB_x in Sephadex LH-20

Fractions: 3rd MEC

Fraction	$\bar{M}n$	Wt (mg)	K_a	K_i
1	2862	98.4	.87	.89
2	2364	209.1	1.18	1.18
3	2209	200.3	1.09	1.26
4	2008	115.5	1.06	1.23
5	1886	43.3	1.02	1.26
6	1466	22.9	.93	.91
7		2.43	.17	
Total Recovery	691.9 mg			
%	101.9			

Table IV

The Weight Percent Distribution of PGB_x in Fractions Separated by Repetitive Sephadex LH-20 MEC

	MEC Run		
Fraction	#1	#2	#3
1	13.1	10.7	14.2
2	19.9	23.8	30.2
3	4.1	27.5	29.0
4	12.1	20.1	16.7
5	18.9	9.7	6.3
6	16.4	6.7	3.3
7	6.6	1.5	.4

Table V

$A_{\lambda 243nm}/A_{\lambda 310nm}$

of Fractions Separated by Repetitive MEC

Fraction	1st MEC	2nd MEC	3rd MEC
1	8.14	10.42	10.22
2	6.85	9.50	9.00
3	5.58	8.08	9.89
4	4.96	7.67	8.60
5	4.12	6.20	7.33
6	3.55	5.05	6.23
7	---	4.33	4.78

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